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Styrylpyrone Derivative (SPD), a Novel DENV-2 NS3, NS5 and prM Inhibitor; Inhibited RNA Replication, Protein Transcription and Production of DENV-2 Progeny Particles

(Sebatian Terbitan Stirilpiron (SPD), Perencat NS3, NS5 dan prM DENV-2 Novel; Merencat Replikasi RNA, Transkripsi Protein dan Penghasilan Zarah Progeni DENV-2)

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ABSTRACT

In the last few decades, dengue has become much more commonplace worldwide, and it is quickly spreading to other countries. As of right now, supportive care is the only available treatment for dengue infection as there are no approved antivirals. Although a DENV vaccine has recently been used in some countries, its indication is limited due to risk of severe dengue in certain individu. The present study was aimed to investigate the effects of styrylpyrone derivative (SPD) extracted from *Goniothalamus umbrosus* against DENV-2 prM, NS3, and NS5 genes and proteins. Gene expression analysis by qRT-PCR was done to investigate the level of gene expression at different DENV-2's replication phases. *In situ* ELISA assay was used to evaluate the effects of SPD treatment on the protein expression of NS3, NS5, and prM. *In silico* molecular docking was used to understand the interactions between peptide with target proteins. SPD inhibited the formation of infectious mature virus particles in the process of DENV-2 replication cycle by modifying the expression of prM gene during the infection. *In situ* ELISA assay of infected cells confirmed that SPD inhibited prM, NS3, and NS5 proteins. Thus, the SPD has the ability to alter DENV-2 replication cycle at the replication phase and during the formation of infectious mature virus particles stage and reduce progeny infectivity. Molecular docking *in silico* analysis confirmed that SPD can interact with all selected virus proteins through hydrogen bonds and other interactions. This study proved that SPD has the potential as anti DENV-2 by interruption expression of DENV-2 NS3, NS5 and prM genes and proteins at RNA replication, protein transcription, virus particles maturation and progeny infectivity phases of viral replication cycle.

Keywords: DENV-2; docking; inhibitors; NS3; NS5; prM

ABSTRAK

Dalam beberapa dekad yang lalu, denggi telah menjadi penyakit yang biasa berlaku di seluruh dunia dan ia mudah merebak. Setakat ini, penjagaan sokongan adalah satu-satunya rawatan yang tersedia untuk merawat jangkitan denggi kerana ketiadaan agent antivirus yang diluluskan. Walaupun setakat ini beberapa negara mula menggunakan vaksin DENV, namun kebolehgunaannya adalah terhad kerana sesetengah individu berisiko mendapat denggi yang teruk. Kajian ini bertujuan untuk menentukan kesan SPD yang diekstrak daripada *Goniothalamus umbrosus* terhadap gen dan protein prM, NS3 dan NS5 virus DENV-2. Analisis pengekspresan gen menggunakan qRT-PCR dilakukan untuk melihat tahap pengekspresan gen pada fasa replikasi DENV-2 yang berbeza. Ujian ELISA *in situ* digunakan untuk melihat kesan rawatan SPD terhadap pengekspresan protein NS3, NS5 dan prM. Pendokan molekul *in silico* digunakan untuk memahami interaksi antara peptida dengan protein sasaran. SPD menghalang pembentukan zarah virus matang berdaya jangkit dalam proses kitaran replikasi DENV-2 dengan mengubah suai pengekspresan gen prM semasa jangkitan. Ujian ELISA *in situ* bagi sel yang dijangkiti telah mengesahkan bahawa SPD merencat protein prM, NS3 dan NS5. Oleh itu, SPD mempunyai keupayaan untuk mengubah kitaran replikasi DENV-2 pada fasa replikasi dan semasa pembentukan peringkat zarah virus matang berdaya jangkit dan mengurangkan kebolehjangkitan progeni. Pendokan molekul *in silico* mengesahkan bahawa SPD boleh berinteraksi dengan semua protein virus terpilih melalui ikatan hidrogen dan interaksi-interaksi lain. Kajian ini menunjukkan bahawa SPD mempunyai potensi sebagai agen anti-DENV-2 dengan mengganggu pengekspresan gen dan protein DENV-2 NS3, NS5 dan prM dalam kitaran replikasi virus ketika replikasi RNA, transkripsi protein, pematangan zarah virus dan kebolehjangkitan progeni.

Kata kunci: DENV-2; NS3; NS5; pendokan; perencat; prM

INTRODUCTION

Dengue is the most important arthropod-borne viral disease in the world caused by the dengue virus (DENV 1-4) transmitted primarily through mosquito vectors, mainly *Aedes aegyptii* and *Aedes albopictus* (Murugesan & Manoharan 2020). The global incidence of dengue has grown dramatically with about half of the world's population now at risk. In 2023, the highest number of dengue cases was documented, impacting over 80 countries across all WHO regions. The year has seen continuous transmission and an unforeseen surge in dengue cases, leading to a record total of more than 6.5 million cases and over 7,300 fatalities related to dengue reported (WHO 2024). Currently, treatment is limited to symptomatic management, with no antiviral treatment for dengue fever. There are two approved vaccines: Sanofi's Dengvaxia and Takeda's Dengue Tetravalent Vaccine. Dengvaxia can reduce the severity of dengue fever in those who have had a prior infection, however, it may increase the risk of severe dengue in those who have not yet been infected (Obi et al. 2021). Takeda Dengue Vaccine (TDV/TAK-003) is a live attenuated tetravalent dengue vaccine that stimulates several immune system components, such as antibodies and immune cells, and helps protects against all four dengue virus strains (LeFevre et al. 2023).

DENV and other Flaviviruses possess three structural proteins essential for the formation of viral progeny: Envelope (E), pre-membrane (prM), and capsid (C). The DENV prM protein is a crucial chaperone for the viral envelope protein, preventing premature fusion with vesicles during viral export. prM molecules in immature particles are cleaved by host proteases, leading to mature fusogenic virions (Zhang et al. 2021). Blockade of prM cleavage would restrict fusion and represents a novel druggable opportunity against DENV. Viral assembly is highly coupled to the endoplasmic reticulum (ER) compartment. Immature virions bud into the ER lumen and acquire a host-derived membrane containing both prM and E viral proteins. Viral progeny then uses the secretory pathway and is released from the infected host cells by exocytosis. During viral exit, immature virions exploit cellular enzymes, namely, furin and/or other furin type proteases, for the cleavage of the membrane-associated prM protein. Host-protease processing of prM into the soluble pr and membrane-associated M is required to allow E to mediate fusion during viral entry in subsequent rounds of infection. Thus, the blockade of prM processing represents an intriguing novel drug target against DENV (Stolp et al. 2015).

The NS3 protein from DENV is a multi-functional protein of 69 kDa, endowed with protease, helicase, and nucleoside 5′-triphosphatase (NTPase) activities. Thus, NS3 is crucial to viral replication and a fascinating target for the creation of targeted antiviral agents (Abdullah et al. 2023). NS5 is the largest (104 kDa) and the most conserved protein of DENV. It is a bifunctional enzyme with a methyltransferase domain (MTase; residues 1-296) at the N-terminal end and an RNA-dependent RNA polymerase (RdRp; residues 320-900) at the C-terminal end. Specifically, residues 320–368 are highly conserved among flaviviruses that have not been found to participate in the ADE phenomenon (Zhang et al. 2019). These residues are also involved in the interaction with NS3 (van den Elsen, Quek & Luo 2021). Previous studies of DENV NS5 focused primarily on its potential as vaccine antigen or antiviral target, as it is the primary target site for anti-DENV T cell-based immune responses in human and DENV NS5 RdRp domain plays an important role in viral RNA replication (Alves et al. 2016).

Natural products have been proven to be important source of lead molecules; many extracts and compounds of plant origin with anti-dengue activity have been reported (Abd Wahab & Ibrahim 2022; Abd Wahab & Ibrahim 2020b; Abd Wahab et al. 2018; Alagarasu et al. 2022; Jayasekara et al. 2024; Rajapakse et al. 2019; Wu et al. 2024). A few antiviral candidates and their specific therapeutic targets have been explored but their biological effects are yet to be disclosed. The DENV E protein, C protein, NS2B/NS3 protease, NS5 RNA-dependent RNA polymerase (RdRp), NS5 methyltransferase (MTase), NS4A, and NS4B have all been recognized as potential targets for antiviral medication development (Li & Kang 2022; Saleem et al. 2019; Shimizu et al. 2019; Yahya, Abd Wahab & Ibrahim 2024). Because of the high prevalence of dengue fever and the lack of a widely applicable vaccine, an efficient antiviral agent to treat DENV infection is urgently needed. In the present study, we found that the styrylpyrone derivative (SPD) isolated from the root of *Goniothalamus umbrosus* has anti-DENV-2 activity. We first determined the effect of SPD treatment on DENV-2 NS3, NS5 and prM genes expression followed by proteins expression analysis using *in situ* ELISA assay and we did *in silico* molecular docking for validation.

MATERIALS AND METHODS

CELLS AND VIRUS

Two types of cell lines were used in this study: *Aedes albopictus* cell line (C6/36 cells) and African green monkey (*Cercopithecus aethiops*) kidney cells (Vero cells). C6/36 cells were maintained in L-15 medium supplemented with 5% fetal bovine serum (FBS) at 28 °C. Vero cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) at 37 °C with 5% CO_2. Dengue virus type-2 (DENV-2) used in this study is a prototype of the New Guinea C strain.

PREPARATION OF SPD

Styrylpyrone derivative (SPD) was isolated from *Goniothalamus umbrosus* using petroleum solvent as described by Abd Wahab and Ibrahim (2020a).

PRM GENE EXPRESSION ANALYSIS USING QUANTITATIVE REAL TIME-POLYMERASE CHAIN REACTION (QRT-PCR)

Cells were seeded in 75 cm^2 flask and incubated at 37 °C with 5% CO_2 until they reached 90% confluent. There were five types of samples involved: 1) cells infected and treated with 0.0025 mg/mL SPD, 2) cells infected without treatment as negative control, 3) cells infected with 200 FFU DENV-2 without treatment as positive control, 4) cells non-infected and treated with 0.0025 mg/mL SPD as toxicity control and 5) cells without infection and SPD treatment as experimental control (Iberahim et al. 2018). Cells designated for infection were inoculated with DENV-2 at 200 FFU. After adsorption period, cells were treated with 0.0025 mg/mL SPD or replaced with new media whereas non infected cells were treated with 0.0025 mg/mL SPD or replaced with new media. All cells were further incubated until harvesting point at 2, 6, 12, 24, 48, 72, 96, and 120 hpi. Cells for respective sample at each time point were harvested by trypsinization, transferred into RNAse free 50 mL tube and centrifuged at 1500 rpm, 4 °C for 5 min. Supernatant was decanted and cell pellet was transferred into 1.5 mL tube. Cell pellet was washed again using media and centrifuged at 1500 rpm, 4 °C for 5 min. The supernatant was discarded and the cell pellet was then used for the RNA isolation process.

NS3, NS5 AND PRM PROTEIN EXPRESSION ANALYSIS

In situ ELISA was conducted based on Antila et al. (2014) with slight modifications. Vero cells at 2×10^4/well were seeded into 96-well round bottom plate and incubated overnight or up to confluency. There were three samples involved: 1) virus-infected cells (200 FFU) and treated with SPD 0.0025 mg/mL, 2) infected cells without treatment, and 3) cells without infection and treated. Cells were infected with the virus and left for one hour for the ad-sorption process. After the adsorption period, the cells in the wells were treated with 0.0025 mg/mL SPD and incubated according to the specified time (2, 6, 12, 24, 48, 72, 96, and 120 hpi). Growth medium was decanted and cells were fixed in 4% paraformaldehyde for 30 min. After fixation, cells were washed with phosphate buffered saline (PBS) two times before being permeabilized with 0.1% Triton-X 100 for 10 min. Cells were washed with PBS + 0.05% Triton-X 100 + 1% FBS (PBTF) two times. Blocking step was done using 10% FBS in PBS for 90-120 min. Primary antibodies were added with antibody dilution ranged in between 1:100 to 1:200 and incubated for 4-5 h. After incubation, the cells were washed with PBTF 2-3 times. Secondary antibody conjugated with horseradish peroxidase (HRP) was added with dilution ranged in between 1:100 and 1:1000 and incubated for 90-120 min. After incubation, cells were washed at least ten times with PBTF. HRP substrate, TMB One solution was added and the color development was measured at 650 nm. Level of targeted protein expression was calculated using the formula:

$$\frac{[a]\text{ODcontrol} - [b]\text{ODtest well} \times 100}{[a]\text{ODcontrol}}$$

where a is the ODcontrol, optical absorbance of cells without infection and treatment; and b is the ODtest well, optical absorbance of cells infected with virus, infected and treated with SPD and cells treated with SPD without infection.

MOLECULAR DOCKING *in silico* ANALYSIS

The docking of the SPD to the DENV-2 NS3, NS5, and prM proteins were performed using the AutoDock Vina program (Othman et al. 2017). The structure of the SPD was retrieved from the ChemSpider database (http://www.chemspider.com) while the three-dimensional structure of the DENV-2 prM protein was retrieved from the Protein Data Bank (PDB entry: 2×bm) (accessed on July 25, 2023).

DATA ANALYSIS

Statistical analysis was completed using Graph Pad Prism for Windows, version 5. All analyses used a 95% confidence interval. The difference obtained was significant if the p value < 0.05.

RESULTS AND DISCUSSION

EFFECT OF SPD TREATMENT ON DENV-2 NS3, NS5 AND PRM GENES EXPRESSION

To study the effect of SPD treatment on the expression of DENV-2 gene transcripts, SPD at a concentration of 0.0025 mg/mL was used. Treatment was given after the adsorption process and RNA total was taken at each time interval of 2, 6, 12, 24, 48, 72, 96, and 120 hpi. The NS3 gene was selected as a target gene to represent the RNA replication, NS5 gene represent the protein transcription and the prM gene was selected as a target gene to represent the progeny maturation phase in the DENV-2 replication cycle. The prM gene is important in the maturation process of viral progeny (Zheng, Umashankar & Kielian 2010). Therefore, this experiment was performed to compare the expression level and expression pattern of the NS3, NS5, and prM genes throughout the infection period. Evaluation of gene expression levels was made relatively by comparing the Ct value of infected cells without treatment with the Ct value of treated infected cells after normalization.

Figure 1 shows the relative gene expression levels of NS3, NS5, and prM genes based on treatment time. Results showed that NS3 gene expression levels in samples treated with SPD were inhibited significantly ($p < 0.05$) starting from 12 hpi and decreased sharply at 24 hpi. NS3 gene expression rate was found to drop sharply from 4.1 ΔΔct at 2 hpi to -1.1 ΔΔct at 12 hpi. The NS3 gene inhibition rate

was very high at 24 hpi which was -8.0 ΔΔct compared to the untreated sample. The rate of inhibition continued to increase until it reached -11.1 ΔΔct at 48 hpi. The decrease in NS3 gene expression was found to be significant at all time intervals of infection. This increase in the percentage of reduction of foci is supported by the decrease in the percentage of inhibition in the time interval test of addition from 2 to 24 hpi. The NS5 gene showed a high expression rate significantly ($p < 0.05$) at the beginning of infection which was 6.3 ΔΔct at 2 hpi. However, the rate of expression decreased sharply up to -1.6 ΔΔct at 24 hpi. This decrease continued until 96 hpi. The decrease in NS5 gene expression was found to be significant at almost all-time intervals of infection. The expression of the prM gene was found to decrease significantly ($p < 0.05$) at the beginning of the infection which was -2.9 ΔΔct compared to the sample without treatment at 2 hpi. Early inhibition of infection can be attributed to interference with attachment activity and virus entry into host cells (Acosta, Talarico & Damonte 2008). Inhibition of prM gene expression was found to increase dramatically at 24 hpi which is -8.0 ΔΔct compared to samples without treatment and then the inhibition decreases after 96 hpi. In this phase, it is estimated that viral activity is focused on the production of viral structural proteins, the process of packaging virions and the release of new progeny (Krol, Brzuska & Szewczyk 2019). Referring to the results from the removal time test, the percentage reduction of the focus increased from 2 up to 24 hpi. This increase in focal reduction percentage is supported by a decrease in inhibition percentage in the addition time test from 2 to 24 hpi.

SPD was found to inhibit the process of attachment, maturation, and release of DENV-2 progeny. It was found that the inhibition pattern of prM gene expression is almost the same as the expression pattern of E gene. This may be due to the interaction between prM protein and E protein in the assembly process of DENV progeny (Sinha et al. 2024). The prM protein is believed to be involved in protecting the E protein from undergoing conformational changes during the maturation pathway in the acidic environment of the trans-Golgi network. The NS5 gene inhibition pattern in the SPD-treated samples was almost identical to the NS3 gene inhibition pattern in the SPD-treated samples. This is because these two genes work together to perform key functions in the DENV replication cycle. Therefore, there is a relationship between these genes which causes the virus replication to decrease after being treated with SPD. In another study, five types of synthetic compounds were found to act against DENV-2 replication by targeting NS3 protease and RdRp NS5 (Yon et al. 2005). The synergistic reaction of inhibiting the activity of these two proteins results in interference with the replication process of DENV-2. Additionally, several studies have demonstrated a relationship between NS5 and NS3 activity. A reaction sequence was detected during genome replication and RNA capping where NS5 stimulates the NTPase and RNA 5'triphosphatase (5'-RTPase) activities of NS3 while NS3 stimulates the GTase activity of NS5 (Issur et al. 2009).

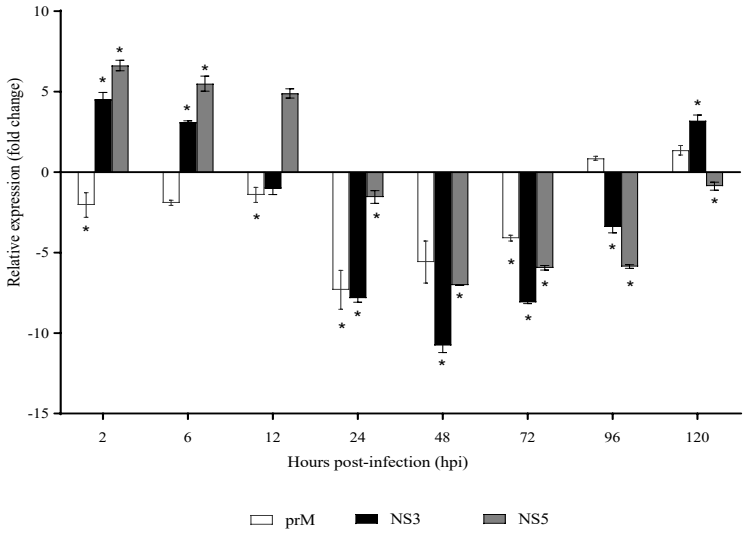

FIGURE 1. Calculated relative gene expression in DENV-2 infected cell treated with SPD relative to the expression of non-treated DENV-2 infected cells for prM, NS3, and NS5 genes at 2, 6, 12, 24, 48, 72, 96, and 120 hpi. At 2, 6, 12, 24, 48 and 72 hpi prM was down-regulated. NS3 was down-regulated at 12, 24, 48, 72, and 96 hpi with obvious downregulation at 48 hpi. NS5 gene was down-regulated starting at 24 hpi until 120 hpi. (*) is significant differences (p-value ≤ 0.05) from DENV-2 infected cells treated with SPD. Control refers to non-treated DENV-2 infected cells with SPD

Previous studies have shown that viruses containing the prM protein are more resistant to acidic environments. During the DENV replication cycle, the prM protein acts as a chaperone that helps ensure proper folding of the E protein. After cleavage of the prM protein, the resulting pr peptide will interact with the E homodimer which in turn stabilizes it and prevents errors during progeny assembly. This indicates an interrelationship between prM and E proteins that may cause their expression patterns to influence each other. E and M proteins are subsequently displayed on the surface of mature DENV. Decreased E gene expression after 24 hpi shows that SPD still acts against the E gene after the maturation process of DENV-2 (Perera & Kuhn 2008).

EFFECT OF SPD TREATMENT ON DENV-2 NS3, NS5 AND PRM PROTEIN EXPRESSION

In situ ELISA assay was performed to understand the effect of SPD on NS3, NS5 and prM protein expression at 2, 6, 12, 24, 48, 72, 96, and 120 hpi. The results of *in situ* ELISA assay showed that the expression of NS3, NS5, and prM protein decreased after being treated with SPD. The rate of expression of prM protein in infected cells treated with SPD was also found to decrease compared to the rate of expression of NS3, NS5, and prM proteins in cells infected without treatment in proportion to treatment time. The results from the *in situ* NS3 protein expression of treated infected cells was found to be decreased when compared to untreated cells. A significant decrease can be seen starting at 24 hpi up to 120 hpi ($p<0.05$) (Figure 2). It was observed that the expression pattern of the NS3 protein almost matched the expression pattern of NS5 protein in untreated infected cells and infected cells treated with SPD. NS5 expression of cells infected without treatment increased proportionally with the time of infection. NS5 protein expression of treated infected cells was found to be decreased when compared to untreated cells (Figure 3). A significant decrease can be seen starting at 24 hpi and also during the next treatment ($p< 0.05$). *In situ* ELISA showed that the expression of the prM protein of the infected and treated cells was decreased when compared to the untreated cells. A significant decrease is observed from 2 hpi up to 120 hpi ($p<0.05$) (Figure 4). The expression level of prM protein was found to be high early in infection and continued to increase up to 120 hpi in untreated cells. The rate of expression of prM protein in infected cells treated with SPD was also found to decrease moderately compared to the rate of expression of prM protein in cells infected without treatment in proportion to treatment time.

It was observed that the expression pattern of NS3 protein was almost identical to the expression pattern of NS5 protein in untreated infected cells and infected cells treated with SPD. According to Johansson et al. (2001), the NS3 protein physically interacts with the NS5 polymerase to ensure that the DENV replication cycle can occur properly. Mutagenesis studies by Matusan et al. (2001) have proven that interference with proteolytic activity or

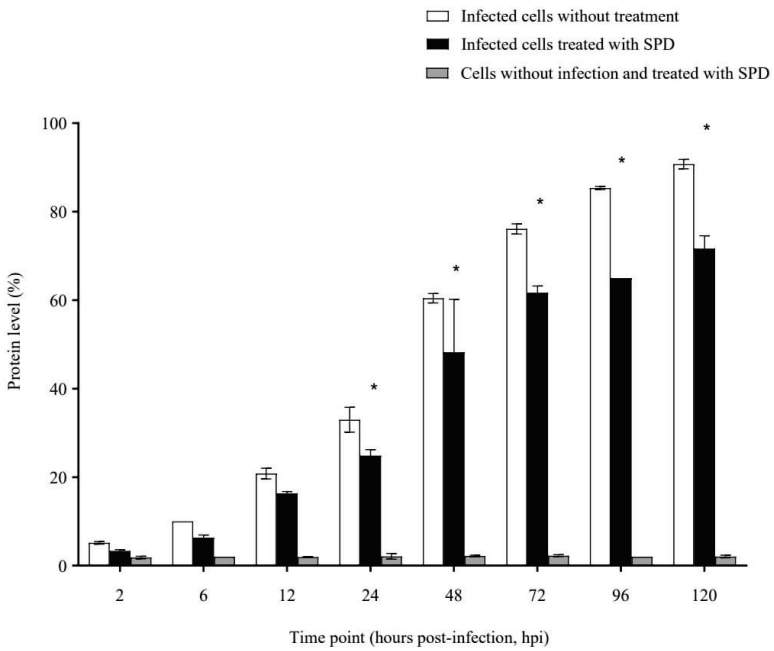

FIGURE 2. Percentage of NS3 protein detected in DENV-2 sample, SPD-DENV-2 sample, and SPD sample across time points. NS3 protein expression was reduced from 2 hpi and have significantly reduced at 24 hpi to 120 hpi in the SPD-DENV-2 sample. (*) represent significant p value < 0.05

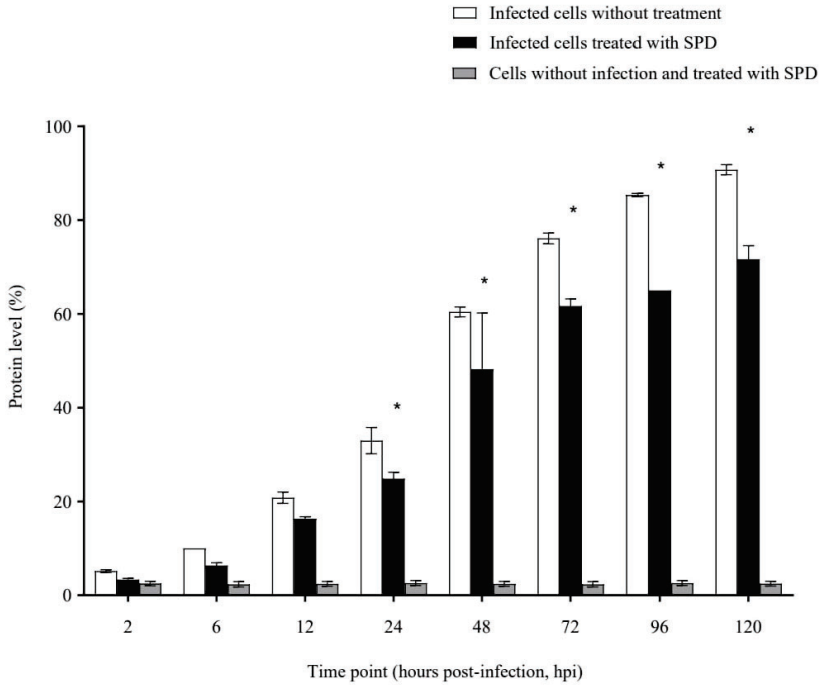

FIGURE 3. Percentage of NS5 protein detected in DENV-2 sample, SPD-DENV-2 sample, and SPD sample across time points. NS5 protein expression was reduced from 2 hpi and have significantly reduced at 24 hpi to 120 hpi in the SPD-DENV-2 sample. (*) represent significant p value < 0.05

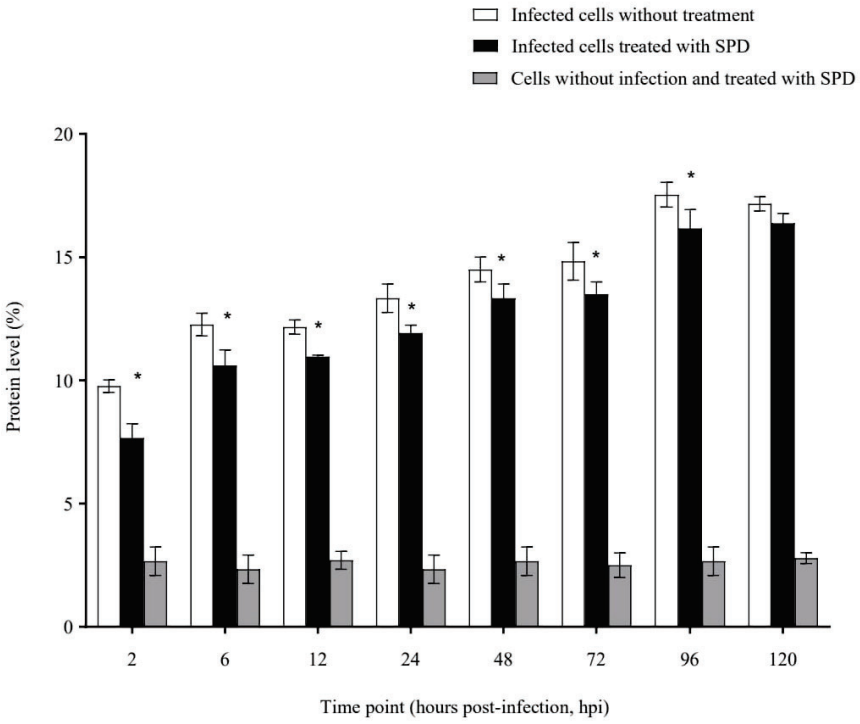

FIGURE 4. Percentage of prM protein detected in DENV-2 sample, SPD-DENV-2 sample, and SPD sample across time points. prM protein expression was significantly reduced from 2 hpi to 120 hpi in the SPD-DENV-2 sample. (*) represent significant p value < 0.05

NTPase/helicase will cause defects in the DENV genome which in turn cause the resulting virus progeny to be non-infectious. Therefore, it is suggested that the inhibitory response of NS3 protein expression by SPD will directly interfere with NS5 protein expression. The NS5 protein is a non-structural protein that has a conserved sequence in DENV. It also has the activity of a bifunctional enzyme which is methyltransferase and RdRp. These enzymes also interact with the NS3 protein (Kapoor et al. 1995). These two proteins work together to perform key functions in the DENV replication cycle. The test results found that the protein expression patterns of NS5 and NS3 were almost the same. It is possible that the inhibitory activity of the NS5 protein by SPD directly interferes with the activity of the NS3 protein. It is known that the interaction between NS3, and NS5 proteins is very important in the DENV replication cycle. This interaction is required in coordinating the synthesis of the positive and negative strands of DENV RNA. This NS3-NS5 interaction can be used as a target in the development of anti-DENV agents (Tay et al. 2015). The results obtained show that SPD is able to cause the expression of these important proteins to be inhibited and subsequently prevent virus replication from occurring.

It is known that the prM protein plays a very important role in DENV infection of host cells. The prM protein is the precursor in the formation of the M protein. Cell proteases will cleave the prM protein to form the mature M protein in the trans Golgi compartment. The intracellular maturation process of DENV will produce many mature and semi-mature virions. Immature virions will also be produced and released from infected cells along with mature and semi-mature virions. These mature or semi-mature DENV particles are also capable of infectivity (Junjhon et al. 2010). This may be the reason why the expression rate of prM protein was found to be high at the beginning of infection and continued to increase up to 120 hpi in untreated cells. This follows that mature, semi-mature, and immature DENV particles will be produced throughout the process of DENV infection on host cells (Rodenhuis-Zybert et al. 2010).

in silico MOLECULAR DOCKING

In silico molecular docking analysis was performed to evaluate the interaction between SPD (ligand) and selected DENV-2 proteins; NS3, NS5, and prM proteins. This information is important to predict the inhibition by SPD of these proteins which at the same time causes the effect of protein destabilization. This analysis is also performed to support the results of gene and protein expression analysis that have been carried out. The results of the *in silico* analysis between SPD and the NS3 protein of DENV-2 (2whx) showed different conformations of SPD with different binding sites on the NS3 protein of DENV-2 (Figure 5). It was found that there was a disruption of the hydrogen bond at ALA164 and LYS88 residues. In addition, there is a pi-alkyl interaction between SPD with ALA164 residues at a bond distance of 4.98 Å and LYS88 at a bond distance of 5.38 Å. SPD has a high affinity value for the NS3 protein because there is more than one bond occurring. The pi-alkyl interaction is a non-covalent interaction that involves the interaction between the pi-electron of the aromatic group and the electron of the alkyl group (Ribas et al. 2022). This interaction involves charge transfer that helps in ligand intercalation at the receptor binding site (Arthur & Uzairu 2019). The average free energy change for SPD interaction with protein NS3 is equal to -4.53 Kcal/mol. These interactions aid the binding of SPD to the NS3 protein of DENV-2 which ultimately inhibits the replication process of DENV-2 (Bernaldez, Billones & Magpantay 2018). The results of this *in silico* molecular docking analysis also support the results of the gene and protein expression analysis that has been done by proving that SPD is able to disrupt the intracellular DENV-2 replication process through the inhibition of NS3 protein activity.

The NS5 protein plays a very important role in DENV replication by activating several important enzymes. This makes NS5 a potential target for DENV inhibitors (Fernandes et al. 2021). The results of the *in silico* molecular docking analysis between SPD and the NS5 protein of DENV-2 (3c6e) gave an average free energy change equal to -4.97 Kcal/mol. The different conformations between SPD and NS5 protein can be seen in Figure 6. Molecular docking results from Autodock Vina show that there is disruption of the hydrogen bond interaction between SPD and the PRO10 and LEU3 residues of the NS5 protein. Alkyl interactions occur between SPD and PRO10 and LEU3 residues respectively at bond distances of 4.24 Å and 3.89 Å making the bond between SPD and NS5 protein stronger. SPD has a high affinity value for the NS5 protein because there is more than one bond occurring. These data support the results of *in vitro* antiviral assays, gene and protein expression analyzes that have been performed showing that intracellular anti-DENV-2 SPD activity can occur effectively even after viral infection into host cells.

In silico molecular docking analysis proved that there is an interaction between SPD and DENV-2 prM protein (2×bm) through hydrogen, van der Waals, pi-alkyl, and pi-sigma bond interactions. The average free energy change for SPD interaction with protein prM is equal to -6.18 Kcal/mol. The different conformations between SPD and prM protein can be seen in Figure 7. The results of the *in silico* molecular docking analysis obtained show that hydrogen bond interactions occur at residues VAL132, ILE147, and LYS105 of DENV-2 prM protein with SPD. Modeling of the prM protein structure of DENV-2 shows that there is van der Waals interaction disruption due to overlapping interactions between residues GLY81, GLY83, HIS110, LYS130, GLU111, THR104, ASP131, and PHE133. If the atoms with van der Waals interactions overlap each other, there is an increase in repulsion energy that can destabilize the protein structure

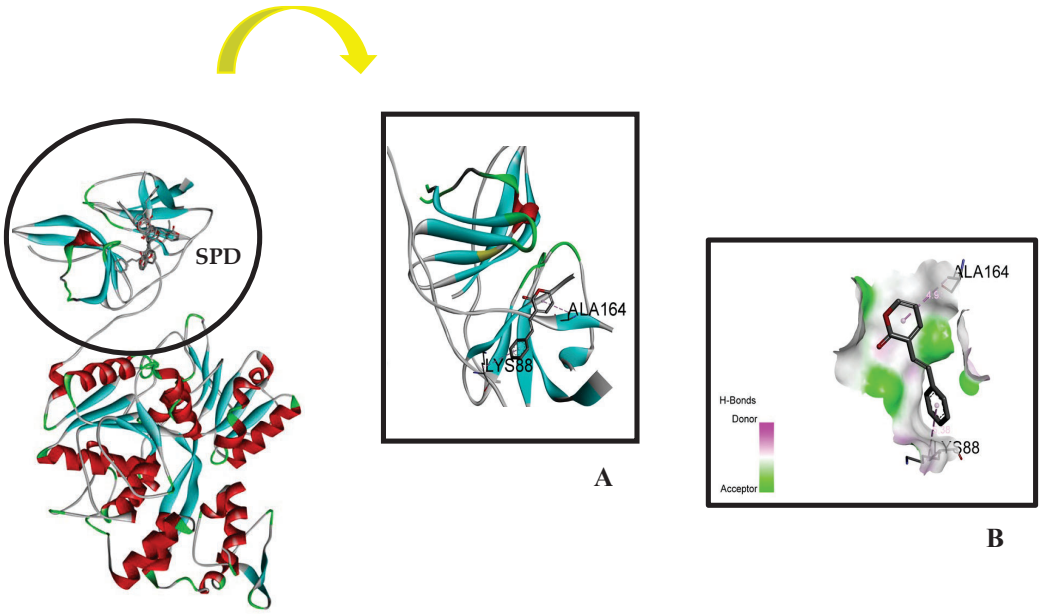

FIGURE 5. Molecular Docking A) Binding pose of SPD molecular structure on DENV-2 NS3 protein (PDB entry: 2whx). The yellow arrow indicates the molecular structure of SPD, and B) Interaction of SPD with the amino acid residues of DENV-2 NS3 protein as shown by docking. SPD has interacted with NS3 protein by forming a hydrogen bond with the amino acid residues of LA164 dan LYS88

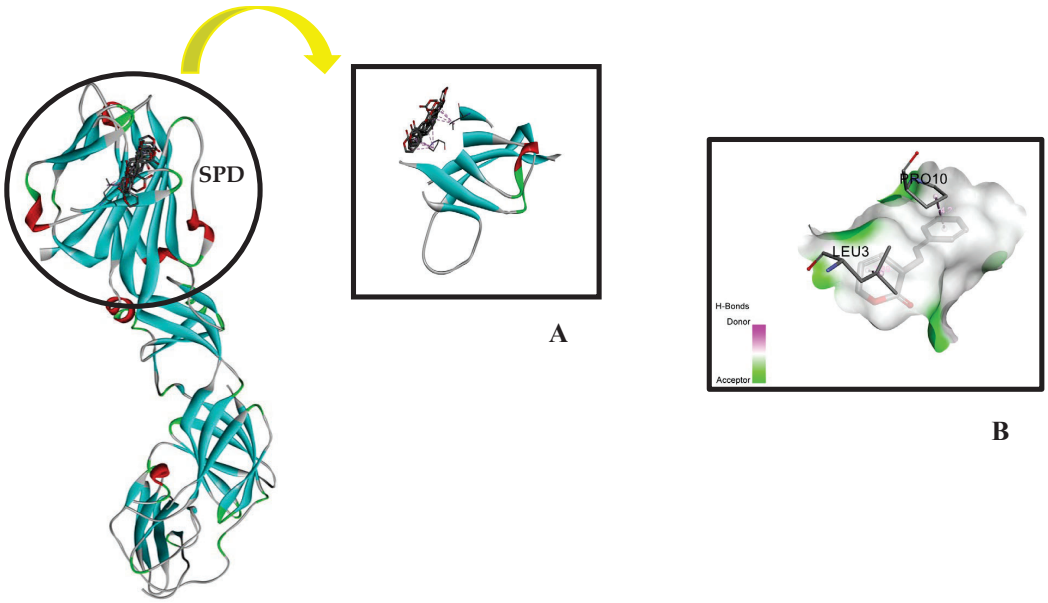

FIGURE 6. Molecular Docking A) Binding pose of SPD molecular structure on DENV-2 NS5 protein (PDB entry: 3c6e). The yellow arrow indicates the molecular structure of SPD, B) Interaction of SPD with the amino acid residues of DENV-2 NS5 protein as shown by docking. SPD has interacted with NS5 protein by forming a hydrogen bond with the amino acid residues of LEU3 dan PRO10

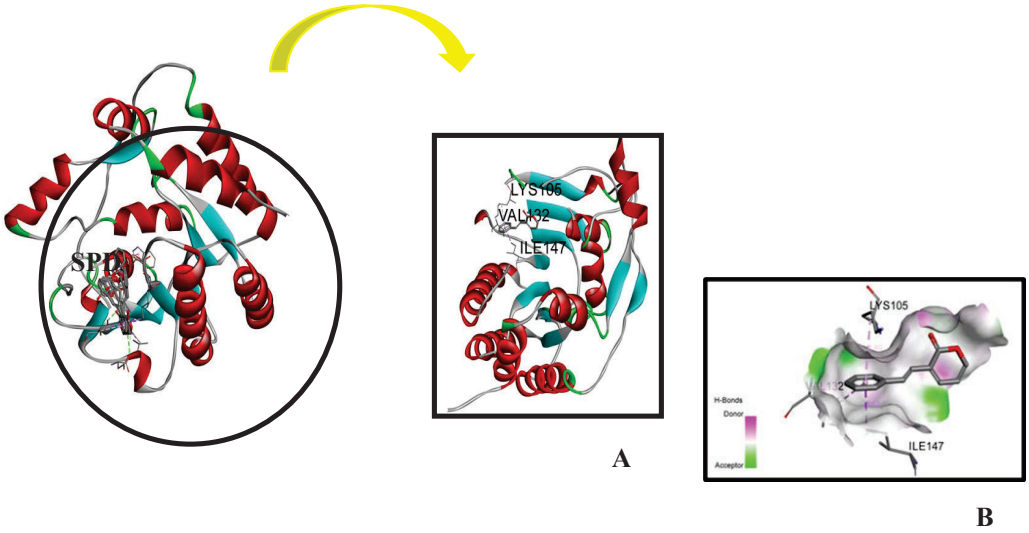

FIGURE 7. Molecular Docking A) Binding pose of SPD molecular structure on DENV-2 prM protein (PDB entry: 2×bm). The yellow arrow indicates the molecular structure of SPD, B) Interaction of SPD with the amino acid residues of DENV-2 prM protein as shown by docking. SPD has interacted with prM protein by forming a hydrogen bond with the amino acid residues of LYS105 dan ILE147

(Berg, Tymoczko & Stryer 2002). In addition, there is a pi-alkyl interaction between SPD with LYS105 and VAL132 residues in DENV-2 prM protein at a distance of 4.40 Å and 4.97 Å, respectively. SPD has a high affinity value for the prM protein because there is more than one bond that occurs. The pi-sigma bond interaction was found to occur between ILE147 residue and SPD at a bond distance of 3.62 Å. These non-covalent interactions are important temporary interactions formed between proteins and ligands where they connect the positive charge of cations with electrons in the pi system of a molecule (Gamez 2014). These interactions strengthen the bond between SPD and the prM protein. This binding can influence the inhibition of prM protein activity.

The maturation process of flavivirus virions is induced by the proteolytic cleavage of the precursor membrane protein (prM) which turns immature virus particles into infectious viruses (Li et al. 2008). Most viruses, including flaviviruses, undergo virion maturation before being released from the host cell. This is to ensure the successful transfer of infectious virions to a new host cell which involves the process of virion attachment, fusion, and entry (Mukhopadhyay, Kuhn & Rossmann 2005). The NS3 protein is a non-structural protein that plays a very important role in the replication and processing of flavivirus polyproteins (Chambers et al. 1990). It has a protease serine domain at the N terminus and its activity depends on the interaction with the NS2B cofactor to form the NS2BNS3 pro-complex. This complex is very important because it has the ability to cut the viral proteins. Any interference with this part will cause inhibition of viral replication. Therefore, this complex is considered an important target in the development of anti-DENV agents (Rothan et al. 2012). The results of *in silico* molecular docking analysis using AutoDock Vina found that SPD showed interaction with the two selected proteins; NS3, and NS5. *In silico* molecular docking analysis conducted on these DENV-2 proteins with SPD suggests there are changes in protein structure that can alter protein stability. The *in silico* molecular docking that has been performed shows that SPD is a potential inhibitor of DENV-2 protein. This is in line with the results gathered in the *in vitro* antiviral test, gene and protein expression analysis that has been carried out. This *in silico* study proved that SPD inhibits the activity of NS3, and NS5 proteins. *In silico* molecular docking analysis is an approach which is good for predicting and matching the desired ligand and protein to know the conformational modification that may occur and further describe the entire interaction associated (Mohamad Yussoff et al. 2020). SPD was identified to arrest the replication cycle of DENV-2 through its inhibitory reaction on NS3, and NS5 genes and proteins.

This study has given further insightful of the action of SPD as an inhibitor against the NS3, and NS5 proteins of DENV-2 which is useful in the evolution of anti-DENV drugs. SPD showed a very good inhibitory effect on the expression of DENV-2 genes and proteins. It is predicted that SPD is also likely to have antiviral activity against DENV-1, DENV3, and DENV-4. Therefore, further studies need to be done to see the antiviral effect of SPD against

DENV-1, DENV-3, and DENV-4 because proteins such as NS3, and NS5 have conserved sequences in all four DENV serotypes. This research can also be continued more deeply through the analysis of the expression of other structural and non-structural genes of DENV-2 as well as the genes of infected host cells. *In vivo* studies using animals as research models should be done because *in vitro* studies alone cannot show the same effect due to differences in terms of metabolic pharmacokinetics between animals and cell cultures.

CONCLUSIONS

We demonstrated that SPD showed a very good inhibitory effect on the expression of NS3, NS5 and prM genes and proteins in DENV-2 infected cells. We have identified the critical interaction between DENV-2 NS3, NS5 and prM genes and proteins gene expression assay, protein expression assay, and *in silico* molecular docking analysis. Thus, we recommend that SPD represents a possible new candidate for the development of anti-dengue therapeutics.

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